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By Ann H. Haffett

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MICHAEL LEBRUN ET AL

SERIAL NO. 08/945,144

INTERNATIONAL APPLN.: PCT/FR96/01125

INTERNATIONAL FILING DATE: 7/18/96

BOX PCT

FILED: OCTOBER 14, 1997

FOR: MUTATED 5-ENOL PYRUVYLSHIKI-
MATE-3-PHOSPHATE SYNTHASE, GENE
CODING FOR SAID PROTEIN AND TRANS-
FORMED PLANTS CONTAINING SAID GENE

Assistant Commissioner for Patents
Washington, D.C. 20231

COMPLETION OF APPLICATION FORMALITIES

Sir:

Enclosed herewith are the following:

☒ A copy of the Notification of Missing Requirements
Under 35 U.S.C. 371 in the United States Designated/Elected Office
(DO/EO/US).

☒ Check for the surcharge (\$130.00) under 37 CFR
1.492(e).

☐ A Petition for Extension under 37 CFR 1.136(a) and
Check for the applicable fee.

☒ An executed Declaration & Power of Attorney, signed
by the inventor(s), which references the application as filed.

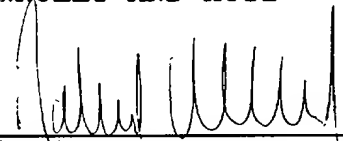
00945144-010000

The Commissioner is hereby authorized to charge any additional fees, or credit any overpayment to, Deposit Account No. 03-2775. This paper is submitted in duplicate.

Respectfully Submitted,

CONNOLLY AND HUTZ

By:



Robert G. McMorrow, Jr.
Registration No. 30,962
Telephone No. (302)658-9141

23858

108 Rec'd PCT/PTO 14 OCT 1997
 Atty. Docket #. 108/100

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

INTERNATIONAL APPL. NO.: PCT/FR96/01125 :
 INTERNATIONAL FILING DATE: 07/18/96 :
 APPLICANT: MICHEL LEBRUN ET AL :
 SERIAL NO: : ART UNIT:
 FILED: : EXAMINER:
 FOR: "MUTATED 5-ENOL PYRUVYLSHIKI- :
 MATE-3-PHOSPHATE SYNTHASE, GENE CODING :
 FOR SAID PROTEIN AND TRANSFORMED :
 PLANTS CONTAINING SAID GENE"

Hon. Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

"Express Mail" No.: EI841020925 US Date: OCTOBER 14, 1997

I hereby certify that this paper, along with any other paper or fee referred to in this paper as being transmitted herewith, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, postage prepaid, on the date indicated above, addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231

-Ann F. Griffith -
 (Typed or printed name of mailing paper or fee)

Ann F. Griffith
 (Signature of person mailing paper)

**TRANSMITTAL OF APPLICATION PAPERS
 TO U.S. DESIGNATED/ELECTED OFFICE (DO/EO/US)
 CONCERNING A FILING UNDER 35 U.S.C. §371
 (CFR 1.494 OR 1.495)**

This Transmittal Letter is based upon PTO Form 1390 (as revised in May, 1993).

The above-identified applicant(s) (jointly with their assignee) have filed an International Application under the P.C.T. and hereby submit(s) to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. §371[f]) at any time rather than delay.
4. ☒ A proper Demand for International Preliminary Examination (IPE) was made to the appropriate Authority (IPEA) by the 19th month from the earliest claimed priority date.
5. ☐ A copy of the International Application as filed (35 U.S.C. §371[c][2]) --
 - a. ☐ is transmitted herewith (required when not transmitted by International Bureau).
 - b. ☐ has been transmitted by the International Bureau. See WIPO Publication
WO 97/04103.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A (verified) translation of the International Application into the English language is enclosed.
7. ☐ Amendments to the (specification and) claims of the International Application under PCT Article 19 (35 U.S.C. 371[c][3])
 - a. ☐ are transmitted herewith (required if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
 - e. ☐ will be submitted with the appropriate surcharge.
8. ☐ A translation of the amendments to the claims (and/or the specification) under PCT Article 19 (35 U.S.C. §371[c][3]) is enclosed or will be submitted with the appropriate surcharge.

9. ☒ An oath or declaration/power of attorney of the inventor(s) (35 U.S.C. §371(c)(4)) **will follow**.
[] and is attached to the translation of (or a copy of) the International Application.
[] and is attached to the substitute specification.
10. ☒ A translation of at least the Annexes to the IPE Report under PCT Article 36 (35 U.S.C. §371(c)(5)) **will follow**.

Items 11. to 16. below concern other document(s) or information included:

11. [] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 is enclosed.
12. ☒ An Assignment **will follow** for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 **will follow**.
13. ☒ A FIRST preliminary amendment is enclosed.
A SECOND or SUBSEQUENT preliminary amendment is enclosed.
14. [] A substitute specification (including claims, abstract, drawing) is enclosed.
15. [] A change of power of attorney and/or address letter is enclosed.
16. ☒ Other items of information:

☒ This application is being filed pursuant to 37 CFR 1.494(c) or 1.495(c), and any missing parts will be filed before expiration of--

[] 22 months from the priority date under 37 CFR 1.494(c), or

☒ 32 months from the priority date under 37 CFR 1.495(c).

☒ The undersigned attorney is authorized by the International applicant and by the inventors to enter the National Phase pursuant to 37 CFR 1.494(c) or 1.495(c).

The following additional information relates to the International Application:

- ☒ Receiving Office: France
- ☒ IPEA (if filing under 37 CFR 1.495): EPO
- ☒ Priority Claim(s) (35 USC §§ 119, 365):
French Appln. 95/08979 filed July 19, 1995.
- ☒ A copy of the International Search Report is

☐ enclosed.

☒ attached to the copy of the English Translation of the
International Application.

- ☐ A copy of the Receiving Office Request Form is enclosed.

The fee calculation is set forth on the next page of this Transmittal Letter.

FEE CALCULATION SHEET

☒ A check in payment of the filing fee, calculated as follows, is attached (37 CFR 1.492).

Basic Fee..... \$ 930.00

Total Number of claims in
excess of (20) times \$22.....

Number of independent claims
in excess of (3) times \$82..... -0-

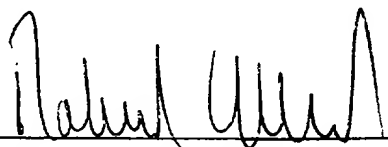
Fee for multiple dependent
claims \$270..... -0-

TOTAL FILING FEE... \$ 930.00

Kindly send us the official filing receipt.

The Commissioner is hereby authorized to charge any additional fees which may be required or to credit any overpayment to Deposit Account No. 03-2775. This is a "general authorization" under 37 CFR 1.25(b), except that no automatic debit of the issue upon allowance is authorized. An additional copy of this page is attached.

Respectfully submitted,

By 

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To Follow:

Declaration
Assignment
Trans. of IPEA Report
into English

RGM/afg
Enclosures

Specification and Claims
Preliminary Amendment
Check for \$930.00
Copy of Notification of Additional Inventor
Copy of Demand (Ch.II)/ 5
Acceptance
IPEA REPORT (In French)
WIPO NOTICE (PCT/IB/332)

RP/PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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MICHEL LEBRUN ET AL :

SERIAL NO: : ART UNIT:

FILED: : EXAMINER:

FOR: "MUTATED 5-ENOL :
PYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE, :
GENE CODING FOR SAID PROTEIN AND :
TRANSFORMED PLANTS CONTAINING SAID :
GENE" :

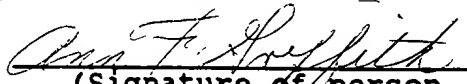
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Washington, D.C. 20231

"Express Mail" No.: EI841020925 Date: OCTOBER 14, 1997

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- ANN F. GRIFFITH -
(Typed or printed name) of
person mailing paper or fee)


(Signature of person
mailing paper or fee)

PRELIMINARY AMENDMENT

Sir:

Prior to any action on the merits of the accompanying new patent application, kindly amend the application as follows:

In the Claims:

Claim 5, lines 1 and 2, change "one of claims 1 to 4" to read -- claim 1 -- ;

RP/PCT

Claim 7, lines 1 and 2, change "one of claims 1 to 4" to read -- claim 1 -- ;

Claim 10, line 6, change "one of claims 1 to 8" to read -- claim 1 -- ;

Claim 13, line 3, change "one of claims 10 to 12" to read -- claim 10 -- ;

Claim 14, lines 2 and 3, change "one of claims 10 to 12" to read -- claim 10 -- ;

Claim 16, line 5, change "one of claims 1 to 8" to read -- claim 1 -- .

R E M A R K S

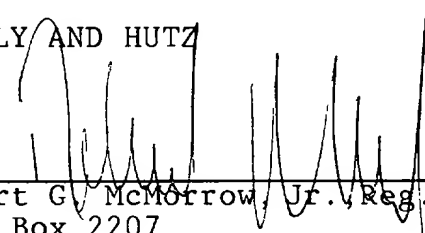
Claims 5, 7, 10, 13, 14 and 16 have been amended to refer to only one preceding claim. Each of the dependent claims, as amended, now depends on only one preceding claim. Therefore no additional fee is required for multiple dependency.

Prompt, favorable action is solicited.

Respectfully submitted,

CONNOLLY AND HUTZ

By


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RGM/afg
(5500*13)

MUTATED 5-ENOL
PYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE,
GENE CODING
FOR SAID PROTEIN
AND TRANSFORMED PLANTS
CONTAINING SAID GENE

Michel Lebrun
Alain Sailland
Georges Freyssinet
and
Eric DeGryse


INTERNATIONAL APPLICATION
IN ENGLISH
including
SEARCH REPORT

RP/PCT

PCT/FR96/01125

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IFD: 07/18/96

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| "Express Mail" mailing label number E1841020925 US |
| Date of Deposit <u>October 14, 1997</u> |
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| ANN F. GRIFFITH |
| (Typed or Printed name of person mailing paper or fee) |
|  |

Mutated 5-enolpyruvylshikimate-3-phosphate synthase,
gene coding for this protein and transformed plants
containing this gene

The present invention relates to a new
5 5-enolpyruvylshikimate-3-phosphate synthase (or EPSPS)
which displays increased tolerance with respect to
herbicides which are competitive inhibitors with
respect to phosphoenolpyruvate (PEP) of EPSPS activity.
This more tolerant EPSP synthase possesses at least one
10 "threonine by isoleucine" substitution. The invention
also relates to a gene coding for such a protein, to
plant cells transformed by chimeric gene constructions
containing this gene, to the plants regenerated from
these cells and also to the plants originating from
15 crossing using these transformed plants.

Glyphosate, sulfosate and fosametine are
broad-spectrum systemic herbicides of the
phosphonomethylglycine family. They act essentially as
competitive inhibitors of 5-enolpyruvylshikimate-3-
20 phosphate synthase (EC 2.5.1.19) or EPSPS with respect
to the PEP (phosphoenolpyruvate). After their
application to the plant, they are translocated in the
plant where they accumulate in the rapidly growing
parts, in particular the cauline and root apices,
25 causing damage to the point of destruction of sensitive
plants.

Plastid EPSPS, the main target of these

products, is an enzyme of the pathway of biosynthesis of aromatic amino acids, which is encoded by one or more nuclear genes and synthesized in the form of a cytoplasmic precursor, then imported into the plastids
5 where it accumulates in its mature form.

The tolerance of plants to glyphosate and to products of the family is obtained by stable introduction into their genome of an EPSPS gene, of plant or bacterial origin, which is mutated or
10 otherwise in respect of the characteristics of inhibition by glyphosate of the product of this gene. In view of the mode of action of glyphosate and the degree of tolerance to glyphosate of the product of the genes which are used, it is advantageous to be able to
15 express the product of the translation of this gene so as enable it to be accumulated in substantial amounts in the plastids.

It is known, for example from US Patent 4,535,060, to confer on a plant a tolerance to a
20 herbicide of the above type, especially N-phosphonomethylglycine or glyphosate, by introducing into the genome of plants a gene coding for an EPSPS carrying at least one mutation that makes this enzyme more resistant to its competitive inhibitor (glyphosate)
25 after localization of the enzyme in the plastid compartment. These techniques, however, need to be improved in order to obtain greater reliability in the use of these plants under agricultural conditions.

In the present description, "plant" is understood to mean any differentiated multicellular organism capable of photosynthesis, and "plant cell" is understood to mean any cell originating from a plant and capable of constituting undifferentiated tissues such as calluses or differentiated tissues such as embryos or plant parts or seeds.

The subject of the present invention is the production of transformed plants having increased tolerance to herbicides of the phosphonemethylglycine family, by regeneration of cells transformed by means of new chimeric genes containing a gene for tolerance to these herbicides.

The subject of the invention is also a chimeric gene for conferring on plants increased tolerance with respect to a herbicide having EPSPS as its target, comprising, in the direction of transcription: a promoter region, optionally a transit peptide region, a sequence of a gene coding for a glyphosate tolerance enzyme and an untranslated polyadenylation signal region at the 3' end, characterized in that the glyphosate tolerance gene contains, relative to the gene from which it is derived, a "threonine 102 by isoleucine" substitution in the "aroA"(EPSPS) region. Preferably, it comprises, in addition, in the same region, a "proline 106 by serine" substitution. These substitutions can be introduced or be present in an EPSPS sequence of any

origin, in particular of plant, bacterial, algal or fungal origin.

The transit peptides which can be used in the transit peptide region can be, known per se, of plant origin, for example originating from maize, sunflower, pea, tobacco or the like. The first and the second transit peptide can be identical, similar or different. They can, in addition, each comprise one or more transit peptide units according to European Patent Application EP 0 508 909. It is the role of this characteristic region to permit the release of a mature and native protein, and especially the above mutated EPSPS, with maximum efficacy in the plasmid compartment.

The promoter region of the chimeric gene according to the invention may be advantageously composed of at least one gene promoter or promoter fragment which is expressed naturally in plants (tubulin, introns, actin, histone).

The untranslated transcription termination signal region at the 3' end of the chimeric gene may be of any origin, for example of bacterial origin, such as that of the nopaline synthase gene, or of plant origin, such as that of the *Arabidopsis thaliana* histone H4A748 gene according to the European Patent Application (European Application 633 317).

The chimeric gene according to the invention can comprise, in addition to the essential portions

above, at least one untranslated intermediate (linker) region, which can be located between the different transcribed regions described above. This intermediate region can be of any origin, for example of bacterial, viral or plant origin.

Isolation of a cDNA coding for a maize EPSPS:

The different steps which led to the obtaining of maize EPSPS cDNA, which served as substrate for the introduction of the two mutations, are described below. All the operations described below are given by way of example, and correspond to a choice made from among the different methods available for arriving at the same result. This choice has no effect on the quality of the result, and consequently any suitable method may be used by a person skilled in the art to arrive at the same result. Most of the methods of engineering of DNA fragments are described in "Current Protocols in Molecular Biology" Volumes 1 and 2, Ausubel F.M. et al., published by Greene Publishing Associates and Wiley-Interscience (1989) (hereinafter, references to protocols described in this work will be designated "ref. CPMB"). The operations relating to DNA which were performed according to the protocols described in this work are especially the following:

ligation of DNA fragments, treatment with Klenow DNA polymerase and T4 DNA polymerase, preparation of plasmid and of bacteriophage λ DNA, either as a minipreparation or as a maxipreparation, and DNA and

RNA analyses according to the Southern and Northern techniques, respectively. Other methods described in this work were followed, and only significant modifications or additions to these protocols have been described below.

Example 1:

1. Obtaining of an *Arabidopsis thaliana* EPSPS fragment

a) Two 20-mer oligonucleotides of respective sequences:

5'-GCTCTGCTCATGTCTGCTCC-3'

5'-GCCCGCCCTTGACAAAGAAA-3'

were synthesized from the sequence of an *Arabidopsis thaliana* EPSPS gene (Klee H.J. et al. (1987) Mol. Gen. Genet., 210, 437-442). These two oligonucleotides are at positions 1523 to 1543 and 1737 to 1717, respectively, of the published sequence, and in opposite orientations.

b) *Arabidopsis thaliana* (var. *columbia*) total DNA was obtained from Clontech (catalogue reference: 6970-1).

c) 50 nanograms (ng) of DNA are mixed with 300 ng of each of the oligonucleotides and subjected to 35 amplification cycles with a Perkin-Elmer 9600 apparatus, under the conditions of standard medium for amplification which are recommended by the supplier. The resulting 204-bp fragment constitutes the

Arabidopsis thaliana EPSPS fragment.

2. Construction of a library of a cDNA from a BMS maize cell line

a) 5 g of filtered cells are ground in liquid nitrogen, and the total nucleic acids are extracted according to the method described by Shure et al. with the following modifications:

- the pH of the lysis buffer is adjusted to pH 9.0;
- after precipitation with isopropanol, the pellet is taken up in water and, after dissolution, adjusted to 2.5 M LiCl. After incubation for 12 h at °C, the pellet from centrifugation for 15 min at 30,000 g at 4°C is resolubilized. The LiCl precipitation step is then repeated. The resolubilized pellet constitutes the RNA fraction of the total nucleic acids.

b) The poly(A)⁺ RNA fraction of the RNA fraction is obtained by chromatography on an oligo(dT)-cellulose column as described in "Current Protocols in Molecular Biology".

c) Synthesis of double-stranded cDNA having a synthetic EcoRI end: this is carried out according to the protocol of the supplier of the different reagents needed for this synthesis in the form of a kit: the "copy kit" from the company In Vitrogen.

Two single-stranded and partially complementary oligonucleotides of respective sequences:

5'-AATTCCTGGG-3'

5'-CTGGG-3' (the latter being

5 phosphorylated)

are ligated with the blunt-ended double-stranded cDNAs.

This ligation of the adaptors results in the creation of SmaI sites attached to the double-stranded cDNAs and EcoRI sites in cohesive form at each end of
10 the double-stranded cDNAs.

d) Creation of the library:

The cDNAs possessing the artificial cohesive EcoRI sites at their ends are ligated with bacteriophage λ gt10 cDNA which has been cut with EcoRI
15 and dephosphorylated according to the protocol of the supplier New England Biolabs.

An aliquot of the ligation reaction was encapsidated in vitro with encapsidation extracts, namely Gigapack Gold, according to the supplier's
20 instructions; this library was titrated using the bacterium *E. coli* C600hfl. The library thereby obtained is amplified and stored according to the instructions of the same supplier, and constitutes the BMS maize cell suspension cDNA library.

25 3. Screening of the BMS maize cell suspension cDNA library with the *Arabidopsis thaliana* EPSP probe

The protocol followed is that of "Current Protocols in Molecular Biology" Volumes 1 and 2,

Ausubel F.M. et al., published by Greene Publishing Associates and Wiley-Interscience (1989) (CPMB).

Briefly, approximately 10^6 recombinant phages are plated out on LB dishes at an average density of 100
 5 phages/cm². The lytic plaques are replicated in duplicate on Amersham Hybond N membranes.

The DNA was fixed to the filters by 1600kJ UV treatment (Stratagene Stratalinker). The filters were prehybridized in 6xSSC/0.1%SDS/0.25 skimmed milk for
 10 2 h at 65°C. The *Arabidopsis thaliana* EPSPS probe was labelled with [³²P]dCTP by random priming according to the supplier's instructions (Pharmacia Ready to Go kit). The specific activity obtained is of the order of
 10⁸ cpm per µg of fragment. After denaturation for 5
 15 min at 100°C, the probe is added to the prehybridization medium and hybridization is continued for 14 hours at 55°C. The filters are fluorographed for 48 h at -80°C with Kodak XAR5 film and Amersham
 Hyperscreen RPN enhancing screens. Alignment of the
 20 positive spots on the filter with the dishes from which they originate enables zones corresponding to the phages displaying a positive hybridization response with the *Arabidopsis thaliana* EPSPS probe to be picked out from the dish. This step of plating out, transfer,
 25 hybridization and recovery is repeated until all the spots in the dish of the successively purified phages prove 100% positive in hybridization. An independent plaque of phage lysis is then picked out in diluent λ

medium (Tris-Cl pH 7.5; 10mM MgSO₄; 0.1M NaCl; 0.1% gelatin); these phages in solution constitute the EPSP-positive clones of the BMS maize cell suspension.

4. Preparation and analysis of the DNA of the 5 EPSP clones of the BMS maize cell suspension

Approximately 5×10^8 phages are added to 20 ml of C600hfl bacteria at an OD_{600nm} value of 2/ml and incubated for 15 minutes at 37°C. This suspension is then diluted in 200 ml of bacterial growth medium in a
10 1-l Erlenmeyer and stirred in a rotary stirrer at 250 rpm. Lysis is noted when the medium clarifies, corresponding to the lysis of the turbid bacteria, and takes place after approximately 4 h of stirring. This supernatant is then treated as described in "Current
15 Protocols in Molecular Biology". The DNA obtained corresponds to the EPSP clones of the BMS maize cell suspension.

One to two µg of this DNA are cut with EcoRI and separated on 0.8% LGTA/TBE agarose gel (ref. CPMB).
20 A final verification consists in checking that the purified DNA does indeed display a hybridization signal with the *Arabidopsis thaliana* EPSPS probe. After electrophoresis, the DNA fragments are transferred onto Amersham Hybond N membranes according to the protocol
25 of Southern described in "Current Protocols in Molecular Biology". The filter is hybridized with the *Arabidopsis thaliana* EPSPS probe according to the conditions described in section 3 above. The clone

displaying a hybridization signal with the *Arabidopsis thaliana* EPSPS probe and containing the longest EcoRI fragment has a size estimated on gel as approximately 1.7 kbp.

5 5. Obtaining of the clone pRPA-ML-711

Ten μ g of the phage clone containing the 1.7-kbp insert are digested with EcoRI and separated on 0.8% LGTA/TBE agarose gel (ref. CPMB). The gel fragment containing the 1.7-kbp insert is excised from the gel
10 by BET staining, and the fragment is treated with β -agarase according to the protocol of the supplier, New England Biolabs. The purified DNA of the 1.7-kbp fragment is ligated at 12°C for 14 h with the DNA of plasmid pUC 19 (New England Biolabs) cut with EcoRI
15 according to the ligation protocol described in "Current Protocols in Molecular Biology". Two μ l of the above ligation mixture are used for the transformation of an aliquot of electrocompetent *E. coli* DH10B; transformation is accomplished by electroporation using
20 the following conditions: the mixture of competent bacteria and ligation medium is introduced into an electroporation cell of thickness 0.2 cm (Biorad) previously cooled to 0°C. The physical conditions of the electroporation using an electroporator made by
25 Biorad are 2500 volts, 25 μ F and 200 Ω . Under these conditions, the mean discharge time of the condenser is of the order of 4.2 milliseconds. The bacteria are then taken up in 1 ml of SOC medium (ref. CPMB) and stirred

for 1 hour at 200 rpm on a rotary stirrer in 15-ml Corning tubes. After plating out on LB/agar medium supplemented with 100 µg/ml of carbenicillin, minipreparations of the bacterial clones which have grown

5 after one night at 37°C are produced according to the protocol described in "Current Protocols in Molecular Biology". After digestion of the DNA with EcoRI and separation by electrophoresis on 0.8% LGTA/TBE agarose gel (ref. CPMB), the clones possessing a 1.7-kbp insert

10 are retained. A final verification consists in checking that the purified DNA does indeed display a hybridization signal with the *Arabidopsis thaliana* EPSPS probe. After electrophoresis, the DNA fragments are transferred onto Amersham Hybond N membranes

15 according to the protocol of Southern described in "Current Protocols in Molecular Biology". The filter is hybridized with the *Arabidopsis thaliana* EPSPS probe according to the conditions described in section 3 above. The plasmid clone possessing a 1.7-kbp insert

20 and hybridizing with the *Arabidopsis thaliana* EPSPS probe was prepared on a larger scale, and the DNA resulting from the lysis of the bacteria was purified on a CsCl gradient as described in "Current Protocols in Molecular Biology". The purified DNA was partially

25 sequenced with a Pharmacia kit according to the supplier's instructions and using as primers the M13 direct and reverse universal primers ordered from the same supplier. The partial sequence produced covers

approximately 0.5 kbp. The derived amino acid sequence in the region of the mature protein (approximately 50 amino acid residues) displays 100% identity with the corresponding amino sequence of mature maize EPSPS described in American Patent USP 4,971,908. This clone, corresponding to a 1.7-kbp EcoRI fragment of the EPSP DNA of the EMS maize cell suspension, was designated pRPA-ML-711. The complete sequence of this clone was determined on both strands using the protocol of the Pharmacia kit and synthesizing complementary oligonucleotides and those of the opposite orientation every 250 bp approximately. The complete sequence obtained of this 1713-bp clone is presented in SEQ ID No. 1.

6. Obtaining of the clone pRPA-ML-715

Analysis of the sequence of the clone pRPA-ML-711, and especially comparison of the derived amino acid sequence with that of maize, shows a sequence extension of 92 bp upstream of the GCG codon coding for the NH₂-terminal alanine of the mature portion of maize EPSPS (American Patent USP 4,971,908). Similarly, an extension of 288 bp downstream of the AAT codon coding for the COOH-terminal asparagine of the mature portion of maize EPSPS (American Patent USP 4,971,908) is observed. These two portions could correspond, in the case of the NH₂-terminal extension to a portion of the sequence of a transit peptide for plastid localization, and, in the case of the COOH-terminal extension, to the

untranslated 3' region of the cDNA.

In order to obtain a cDNA coding for the mature portion of the maize EPSPS cDNA, as described in USP 4,971,908, the following operations were carried out:

a) Removal of the untranslated 3' region:
construction of pRPA-ML-712:

The clone pRPA-ML-711 was cut with the restriction enzyme *AseI*, and the ends resulting from this cleavage were rendered blunt by treatment with the Klenow fragment of DNA polymerase I according to the protocol described in CPMB. A cleavage with the restriction enzyme *SacII* was then performed. The DNA resulting from these operations was separated by electrophoresis on 1% LGTA/TBE agarose gel (ref. CPMB).

The gel fragment containing the 0.4-kbp "AseI-blunt ends/SacII" insert was excised from the gel and purified according to the protocol described in section 5 above. The DNA of the clone pRPA-ML-711 was cut with the restriction enzyme *HindIII* at the *HindIII* site located in the polylinker of the cloning vector pUC19, and the ends resulting from this cleavage were rendered blunt by treatment with the Klenow fragment of DNA polymerase I. A cleavage with the restriction enzyme *SacII* was then performed. The DNA resulting from these manipulations was separated by electrophoresis on 0.7% LGTA/TBE agarose gel (ref. CPMB).

The gel fragment containing the approximately

3.7-kbp HindIII-blunt ends/SacII insert was excised from the gel and purified according to the protocol described in section 5 above.

The two inserts were ligated, and 2 μ l of the ligation mixture were used to transform *E. coli* DH10B as described above in section 5.

The plasmid DNA content of different clones was analysed according to the procedure described for pRPA-ML-711. One of the plasmid clones selected contains an approximately 1.45-kbp EcoRI-HindIII insert. The sequence of the terminal ends of this clone reveals that the 5' end of the insert corresponds exactly to the corresponding end of pRPA-ML-711, and that the 3'-terminal end possesses the following sequence:

"5'-...AATTAAGCTCTAGAGTCGACCTGCAGGCATGCAAGCTT-3'".

The underlined sequence corresponds to the codon of the COOH-terminal amino acid asparagine, the next codon corresponding to the translation stop codon. The nucleotides downstream correspond to sequence elements of the pUC19 polylinker. This clone comprising the pRPA-ML-711 sequence up to the translation termination site of mature maize EPSPS and followed by sequences of the pUC 19 polylinker up to the HindIII site was designated pRPA-ML-712.

b) Modification of the 5' end of pRPA-ML-712: construction of pRPA-ML-715;

The clone pRPA-ML-712 was cut with the

restriction enzymes PstI and HindIII. The DNA resulting from these manipulations was separated by electrophoresis on 0.8% LGTA/TBE agarose gel (ref. CPMB). The gel fragment containing the 1.3-kbp PstI-EcoRI insert was excised from the gel and purified according to the protocol described in section 5 above. This insert was ligated in the presence of an equimolecular amount of each of the two partially complementary oligonucleotides of sequence:

10 Oligo 1: 5'-GAGCCGAGCTCCATGGCCGGCGCCGAGGAGATCGTGCTGCA-3'

Oligo 2: 5'-GCACGATCTCCTCGGCGCCGGCCATGGAGCTCGGCTC-3'

as well as in the presence of plasmid pUC19 DNA digested with the restriction enzymes BamHI and HindIII.

15 Two μ l of the ligation mixture were used to transform *E. coli* DH10B as described above in section 5. After analysis of the plasmid DNA content of different clones according to the procedure described above in section 5, one of the clones possessing an

20 approximately 1.3-kbp insert was retained for subsequent analyses. The sequence of the 5'-terminal end of the selected clone reveals that the DNA sequence in this region is the following: sequence of the pUC19 polylinker from the EcoRI to the BamHI sites, followed

25 by the sequence of the oligonucleotides used in the cloning, followed by the remainder of the sequence present in pRPA-ML-712. This clone was designated pRPA-ML-713. This clone possesses a methionine ATG codon

included in an NcoI site upstream of the N-terminal alanine codon of mature EPSP synthase. Furthermore, the alanine and glycine codons of the N-terminal end have been preserved, but modified on the third variable
5 base: initial GCGGGT gives modified GCCGGC.

The clone pRPA-ML-713 was cut with the restriction enzyme HindIII, and the ends of this cleavage were rendered blunt by treatment with the Klenow fragment of DNA polymerase I. A cleavage with
10 restriction enzyme SacI was then performed. The DNA resulting from these manipulations was separated by electrophoresis on 0.8% LGTA/TBE agarose gel (ref. CPMB). The gel fragment containing the 1.3-kbp "HindIII-blunt ends/SacI" insert was excised from the
15 gel and purified according to the protocol described in section 5 above. This insert was ligated in the presence of plasmid pUC19 DNA digested with restriction enzyme XbaI, and the ends of this cleavage were rendered blunt by treatment with the Klenow fragment of
20 DNA polymerase I. A cleavage with the restriction enzyme SacI was then performed. Two μ l of the ligation mixture were used to transform *E. coli* DH10B as described above in section 5. After analysis of the plasmid DNA content of different clones according to
25 the procedure described above in section 5, one of the clones possessing an approximately 1.3-kbp insert was retained for subsequent analyses. The sequence of the terminal ends of the selected clone reveals that the

DNA sequence is the following: sequence of the pUC19 polylinker from the EcoRI to SacI sites, followed by the sequence of the oligonucleotides used in the cloning from which the 4 bp GATCC of the

5 oligonucleotide 1 described above have been deleted, followed by the remainder of the sequence present in pRPA-ML-712 up to the HindIII site and sequence of the pUC19 polylinker from XbaI to HindIII. This clone was designated pRPA-ML-715.

10 7. Obtaining of a cDNA coding for a mutated maize EPSPS

All the mutagenesis steps were carried out with the Pharmacia U.S.E. mutagenesis kit according to the supplier's instructions. The principle of this
15 mutagenesis system is as follows: plasmid DNA is denatured by heat and reassociated in the presence of a molar excess of, on the one hand the mutagenesis oligonucleotide, and on the other hand an oligonucleotide enabling a unique restriction enzyme site present in
20 the polylinker to be eliminated. After the reassociation step, synthesis of the complementary strand is carried out by the action of T4 DNA polymerase in the presence of T4 DNA ligase and gene 32 protein in a suitable buffer which is supplied. The
25 synthesis product is incubated in the presence of the restriction enzyme for which the site is assumed to have disappeared by mutagenesis. The *E. coli* strain possessing, in particular, the mutS mutation is used as

host for the transformation of this DNA. After growth in liquid medium, the total plasmid DNA is prepared and incubated in the presence of the restriction enzyme used before. After these treatments, *E. coli* strain
 5 DH10B is used as host for the transformation. The plasmid DNA of the clones isolated is prepared, and the presence of the mutation introduced is verified by sequencing.

 A)- modification of sites or sequences
 10 without in principle affecting the EPSPS-resistance character of maize to products which are competitive inhibitors of EPSP synthase activity: elimination of an internal NcoI site from pRPA-ML-715.

 The pRPA-ML-715 sequence is numbered
 15 arbitrarily by placing the first base of the N-terminal alanine codon GCC at position 1. This sequence possesses an NcoI site at position 1217. The site-modification oligonucleotide possesses the sequence:

 5'-CCACAGGATGGCGATGGCCTTCTCC-3'.

20 After sequencing according to the references given above, the sequence read after mutagenesis corresponds to that of the oligonucleotide used. The NcoI site has indeed been eliminated, and the translation into amino acids in this region preserves the initial
 25 sequence present in pRPA-ML-715.

 This clone was designated pRPA-ML-716.

 The 1340-bp sequence of this clone is presented in SEQ ID No. 2 and SEQ ID No. 3.

B)- sequence modifications enabling the EPSPS-resistance character of maize to products which are competitive inhibitors of EPSP synthase activity to be increased.

5 The following oligonucleotides were used:

a) mutation Thr 102 → Ile.

5'-GAATGCTGGAATCGCAATGCGGCCATTGACAGC-3'

b) mutation Pro 106 → Ser.

5'-GAATGCTGGAAGTCAATGCGGTCCTTGACAGC-3'

10 c) mutations Gly 101 → Ala and Thr 102 → Ile.

5'-CTTGGGGAATGCTGCCATCGCAATGCGGCCATTG-3'

d) mutations Thr 102 → Ile and Pro 106 → Ser.

5'-GGGGAATGCTGGAATCGCAATGCGGTCCTTGACAGC-3'

15 After sequencing, the sequence read after mutagenesis on the three mutated fragments is identical to the parent pRPA-ML-716 DNA sequence, with the exception of the mutagenized region which corresponds to that of the mutagenesis oligonucleotides used. These clones were designated: pRPA-ML-717 for the mutation
20 Thr 102 → Ile, pRPA-ML-718 for the mutation Pro 106 → Ser, pRPA-ML-719 for the mutations Gly 101 → Ala and Thr 102 → Ile and pRPA-ML-720 for the mutations Thr 102 → Ile and Pro 106 → Ser.

The 1340-bp sequence of pRPA-ML-720 is

presented in SEQ ID No. 4 and SEQ ID No. 5.

The 1395-bp NcoI-HindIII insert is the basis of all the constructions used for the transformation of plants for the introduction of resistance to herbicides which are competitive inhibitors of EPSPS, and especially glyphosate resistance. This insert will be designated in the remainder of the description "the maize EPSPS double mutant".

Example 2:

10 Glyphosate tolerance of the different mutants in vitro

2.a: Extraction of EPSP synthase

The different EPSP synthase genes are introduced in the form of an NcoI-HindIII cassette into the plasmid vector pTc99a (Pharmacia, ref: 27-5007-01) cut with NcoI and HindIII. Recombinant *E. coli* DH10B bacteria overexpressing the different EPSP synthases are sonicated in 40 ml of buffer per 10 g of pelleted cells, and washed with this same buffer (200 mM Tris-HCl pH 7.8, 50 mM mercaptoethanol, 5 mM EDTA and 1 mM PMSF), to which 1 g of polyvinylpyrrolidone is added. The suspension is stirred for 15 minutes at 4°C and then centrifuged for 20 minutes at 27,000 g and 4°C.

Ammonium sulphate is added to the supernatant to bring the solution to 40% saturation with respect to ammonium sulphate. The mixture is centrifuged for 20 minutes at 27,000 g and 4°C. Ammonium sulphate is added to the new supernatant to bring the solution to

70% saturation with respect to ammonium sulphate. The mixture is centrifuged for 30 minutes at 27,000 g and 4°C. The EPSP synthase present in this protein pellet is taken up in 1 ml of buffer (20 mM Tris-HCl pH 7.8 and 50 mM mercaptoethanol). This solution is dialysed overnight against two litres of this same buffer at 4°C.

2.b: Enzyme activity

The activity of each enzyme, as well as its glyphosate resistance, is measured in vitro over 10 minutes at 37°C in the following reaction mixture: 100 mM maleic acid pH 5.6, 1 mM phosphoenolpyruvate, 3 mM shikimate 3-phosphate (prepared according to Knowles P.F. and Sprinson D.B. 1970. Methods in Enzymol 17A, 351-352 from *Aerobacter aerogenes* strain ATCC 25597) and 10 mM potassium fluoride. The enzyme extract is added at the last moment after the addition of glyphosate, the final concentration of which varies from 0 to 20 mM.

The activity is measured by assaying the phosphate liberated according to the technique of Tausky H.A. and Shorr E. 1953. J. Biol. Chem. 202, 675-685.

Under these conditions, the wild-type (WT) enzyme is already 85% inhibited at a glyphosate concentration of 0.12 mM. At this concentration, the mutant enzyme known as Ser106 is only 50% inhibited, and the other three mutants, Ile102, Ile102/Ser106 and

Ala101/Ile102, show little or no inhibition.

The glyphosate concentration has to be multiplied by ten, that is to say 1.2 mM, in order to produce a 50% inhibition of the mutant enzyme Ile102, the mutants Ile102/Ser106, Ala/Ile and Ala still not being inhibited.

It should be noted that the activity of the mutants Ala/Ile and Ala is not inhibited up to glyphosate concentrations of 10mM, and that that of the mutant Ile102/Ser106 is not reduced even if the glyphosate concentration is multiplied by 2, that is to say 20 mM.

Example 3:

Resistance of transformed tobacco plants

1-1- Transformation

The vector pRPA-RD-173 is introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al., 1986). The transformation technique is based on the procedure of Horsh et al. (1985).

1-2- Regeneration

The regeneration of PBD6 tobacco (source SEITA France) from leaf explants is carried out on a Murashige and Skoog (MS) basal medium comprising 30 g/l of sucrose as well as 200 µg/ml of kanamycin. The leaf explants are removed from plants cultivated in the greenhouse or in vitro and are transformed according to the leaf disc technique (Science, 1985, Vol. 227, pp.

1229-1231) in three successive steps: the first comprises the induction of shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The shoots formed during this step are then developed for 10 days by culturing on an MS medium supplemented with 30 g/l of sucrose but not containing any hormone. Shoots which have developed are then removed and cultured on an MS rooting medium having half the content of salts, vitamins and sugar and not containing any hormone. After approximately 15 days, the rooted shoots are transferred to soil.

1-3- Glyphosate resistance

Twenty transformed plants were regenerated and transferred to the greenhouse for the construction of pRPA-RD-173. These plants were treated in the greenhouse at the 5-leaf stage with an aqueous suspension of RoundUp corresponding to 0.8 kg of glyphosate active substance per hectare.

The results correspond to the observation of phytotoxicity indices recorded 3 weeks after treatment. Under these conditions, it is found that the plants transformed with the construction pRPA-RD-173 display very good tolerance, whereas the untransformed control plants are completely destroyed.

These results show clearly the improvement brought about by the use of a chimeric gene according

to the invention for the same gene coding for glyphosate tolerance.

Example 4:

Transformation and selection of maize cells

5 BMS (Black Mexican Sweet) maize cells in an exponential growth phase are bombarded with the construction pRPA-RD-130 according to the principle and the protocol described by Klein et al. 1987 (Klein T.M., Wolf E.D., Wu R. and Sandford J.C. (1987): High
10 velocity microprojectiles for delivering nucleic acids into living cells, NATURE Vol. 327 pp. 70-73).

Two days after bombardment, the cells are transferred to the same medium containing 2 mM N-(phosphonomethyl)glycine.

15 After 8 weeks of selection on this medium, calluses which develop are selected, then amplified and analysed by PCR, and reveal clearly the presence of the chimeric OTP-EPSPS gene.

20 Cells not bombarded and grown on the same medium containing 2 mM N-(phosphonomethyl)glycine are blocked by the herbicide and do not develop.

 The transformed plants according to the invention may be used as parents for obtaining lines and hybrids having the phenotypic character
25 corresponding to the expression of the chimeric gene introduced.

Description of the constructions of the
plasmids

pRPA-RD-124: Addition of a "nos"

polyadenylation signal to pRPA-ML-720 with creation of
5 a cloning cassette containing the maize double mutant
EPSPS gene (Thr 102 → Ile and Pro 106 → Ser). pRPA-ML-
720 is digested with HindIII and treated with the
Klenow fragment of *E. coli* DNA polymerase I to produce
a blunt end. A second digestion is performed with NcoI,
10 and the EPSPS fragment is purified. The EPSPS gene is
then ligated with purified pRPA-RD-12 (a cloning
cassette containing the polyadenylation signal of
nopaline synthase) to give pRPA-RD-124. To obtain the
useful purified vector pRPA-RD-12, it was necessary for
15 the latter to be digested beforehand with SalI, treated
with Klenow DNA polymerase and then digested a second
time with NcoI.

pRPA-RD-125: Addition of an optimized transit
peptide (OTP) to pRPA-RD-124 with creation of a cloning
20 cassette containing the EPSPS gene targeted on the
plasmids. pRPA-RD-7 (European Patent Application
EP 652 286) is digested with SphI, treated with T4 DNA
polymerase and then digested with SpeI, and the OTP
fragment is purified. This OTP fragment is cloned into
25 pRPA-RD-124 which has previously been digested with
NcoI, treated with Klenow DNA polymerase to remove the
protruding 3' portion and then digested with SpeI. This
clone is then sequenced in order to ensure correct

translational fusion between the OTP and the EPSPS gene. pRPA-RD-125 is then obtained.

pRPA-RD-130: Addition of the H3C4 maize histone promoter and of adh1 intron 1 sequences of pRPA-RD-123 (Patent Application EP 507 698) to pRPA-RD-125 with creation of a cassette for expression in plants for the expression of the double mutant EPSPS gene in the tissues of monocotyledons. pRPA-RD-123 (a cassette containing the H3C4 maize histone promoter fused with the adh1 intron 1) is digested with NcoI and SacI. The DNA fragment containing the promoter derived from pRPA-RD-123 is then purified and ligated with pRPA-RD-125 which has previously been digested with NcoI and SacI.

pRPA-RD-159: Addition of the H4A748 Arabidopsis histone double promoter (Patent Application EP 507 698) to pRPA-RD-125 with creation of a cassette for expression in plants for the expression of the "OTP-double mutant EPSPS gene" gene in the tissues of dicotyledons. pRPA-RD-132 (a cassette containing the H4A748 double promoter (Patent Application EP 507 698)) is digested with NcoI and SacI. The purified promoter fragment is then cloned into pRPA-RD-125 which has been digested with EcoI and SacI.

pRPA-RD-173: Addition of the "H4A748 promoter-OTP-double mutant EPSPS gene" gene of pRPA-RD-159 to plasmid pRPA-BL-150A (European Patent Application 508 909) with creation of an Agrobacterium

tumefaciens transformation vector. pRPA-RD-139 is digested with NotI and treated with Klenow polymerase. This fragment is then cloned into pRPA-BL-150A with SmaI.

(1) GENERAL INFORMATION:

- (i) APPLICANT: Lebrun, Michel
Sailland, Alain
Freyssinet, Georges
DeGryse, Eric
- (ii) TITLE OF INVENTION: Mutated 5-enolpyruvylshikimate-3-phosphate synthase,
gene coding for this protein and transformed plants containing this gene
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Cornolli, Bove, Lodge and Hutz
(B) STREET: 1220 Market Street
(C) CITY: Wilmington
(D) STATE: Delaware
(E) COUNTRY: U.S.A.
(F) ZIP: 19899
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE:
(B) COMPUTER:
(C) OPERATING SYSTEM:
(D) SOFTWARE:
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/FR96/01125
(B) FILING DATE: 18-JUL-1996
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Robert G. McMorow, Jr.
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (302) 658-9141
(B) TELEFAX: (302) 658-5614

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1713 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) ORIGINAL SOURCE:
(A) ORGANISM: See says
(B) STRAIN: Black Mexican Sweet
(F) TISSUE TYPE: Callus
- (iv) IMMEDIATE SOURCE:
(A) LIBRARY: lambda gt10
(B) CLONE: pAPA-ML-711

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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| CCATCAAGGA GATCTCGGC ACCGTCAAGC TCCCGCGTGC CAAGTGCCTT TCCACCGGA | 180 |
| TCTCTACTCT GCGCGCGCTG TCCGAGGGA CAACATGTGT TGATAAGCTG CTGACAGTGC | 240 |
| AGGATGTCCA CTACATGCTC GCGCGCTTGA GACTCTTGG TCTCTCTGTC GAAGCGGACA | 300 |
| AAGCTGCCCA AAGAGCTGTA GTTGTGTGGT GTGTGTGAAA GTTCCCATTT GAGGAAGCTA | 360 |
| AAGAGGAAGT GCACTCTTTC TTGGGGAATG CTGGAAGTGC AATCCCGCCA TTGACAGCAG | 420 |
| CTGTACTCTC TCTGTGTGGA AATCAACTT ACCTGCTTGA TGAATACCA AGAATGAGGG | 480 |

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ATGAGCCGAT TGGGACCTTG GTTGTGATAT TGAAGCAACT TGTTCAGAT GTTGATGTT 540
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TTTGTGCTG TGGGATG GAGATGAAA TCATTGATAA ATTAATGCTG ATTGCTGCTG 720
TGGAAATGAC ATTGAGATG ATGAGGCTT TTGTTGTGAA AGCAGAGCAT TGTGATAGT 780
GGGACAGATT CTACATTAAG GGAGGTGAAA AATACAGTC CCTAAAAAT GCTATGTTG 840
AAGGTGATGC CTCAGGCA AGCTATTCT TGGCTGCTG TCGAATTACT GGAGGGACTG 900
TGAATGTGA AGTTGTGCT ACCAGCACTT TGCAGGCTG TGTGAATTT GCTGAGGTAC 960
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CGCGGGAGCC ATTTGGGAGG AAACAGCTCA AGCGATGGA TGTCAACATG AACAGATGC 1080
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CCTTGGCGA CTACTTGAT GTCTGAGCA CTTCTGCTA GAATTAATAA AGCTGCGAT 1440
ACTACGAGC AGCTGATG AGTGATAGG CTGTGCTGA GGAAATACAT TTCTTTGTT 1500
CTGTTTTCT CTTGACGGG ATTAAGTTT GAGTCTGAA CTTAGTTGT TTGTAGCAG 1560
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AAAAAAAAA AAAAAAAAAA AACCGGGAA TTC 1713

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1340 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Zea mays
- (B) STRAIN: Black Mexican Sweet

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pRPA-ML-716

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 6..1337

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

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1 5 10

TCC GGC ACC GTC AAG CTG CCG GGG TCC AAG TCG CTT TCC AAC CCG ATC 95
Ser Gly Thr Val Lys Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile
15 20 25 30

CTC CTA CTC GGC GGC CTG TCC GAG GGG ACA ACA GTG GTT GAT AAC CTG 143
Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu
35 40 45

CTG AAC AGT GAG GAT GTC CAC TAC ATG CTC GGG GGC TTG AGG ACT CTT 191
Leu Asn Ser Glu Asp Val His Tyr Met Leu Gly Ala Leu Arg Thr Leu

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| 50 | 55 | 60 | |
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| AAA GCA GAG CAT TCT GAT AGC TGG GAC AGA TTC TAC ATT AAG GGA GGT Lys Ala Glu His Ser Asp Ser Trp Asp Arg Phe Tyr Ile Lys Gly Gly 225 230 235 | | | 719 |
| CAA AAA TAC AAG TCC CCT AAA AAT GGC TAT GTT GAA GGT GAT GGC TCA Gln Lys Tyr Lys Ser Pro Lys Asn Ala Tyr Val Glu Gly Asp Ala Ser 240 245 250 | | | 767 |
| AGC GCA AGC TAT TTC TTG GCT GGT GCT GCA ATT ACT GGA GGG ACT GTG Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Val 255 260 265 270 | | | 815 |
| ACT GTG GAA GGT TGT GGC ACC ACC AGT TTG CAG GGT GAT GTG AAG TTT Thr Val Glu Gly Cys Gly Thr Thr Ser Leu Gln Gly Asp Val Lys Phe 275 280 285 | | | 863 |
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| AGC GTA ACT GTT ACT GGC CCA CCG CCG GAG CCA TTT GAG AGG AAA CAC Ser Val Thr Val Thr Gly Pro Pro Arg Glu Pro Phe Gly Arg Lys His 305 310 315 | | | 959 |
| CTC AAG GCG ATT GAT GTC AAG ATG AAC AAG ATG CCT GAT GTC GGC ATG Leu Lys Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met 320 325 330 | | | 1007 |
| ACT CTT GCT GTG GTT GGC CTC TTT GGC GAT GGC CCG ACA GGC ATC AGA Thr Leu Ala Val Val Ala Leu Phe Ala Asp Gly Pro Thr Ala Ile Arg 335 340 345 350 | | | 1055 |
| GAC GTG GCT TCC TGG AGA GTA AAG GAG ACC GAG AGG ATG GTT GCG ATC Asp Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Val Ala Ile 355 360 365 | | | 1103 |
| CGG ACC GAG CTA ACC AAG CTG GGA GCA TCT GTT GAG GAA GGG CCG GAC Arg Thr Glu Leu Thr Lys Leu Gly Ala Ser Val Glu Glu Gly Pro Asp 370 375 380 385 390 | | | 1151 |

| J70 | J75 | J80 | |
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| TAC TGC ATC ATC ACU CCG CCG GAG AAG GTG AAC GTG ACC GCG ATC GAC Phe Cys Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp 384 390 396 | | | 1199 |
| ACG TAC GAG GAG CAG AGG ATG GCG ATG GCG TTC TCG CTT GCG GCG TGT Thr Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys 400 405 410 | | | 1217 |
| GCG GAG GTG CCG GTG ACC ATC CCG GAC CCG GCG TGC ACC CCG AAG ACC Ala Glu Val Pro Val Thr Ile Arg Asp Pro Gly Cys Thr Arg Lys Thr 415 420 425 430 | | | 1295 |
| TTC CCG GAC TAC TTC GAT GTG CTG AGC ACT TTC GTC AAG AAT Phe Pro Asp Tyr Phe Asp Val Leu Ser Thr Phe Val Lys Asn 435 440 | | | 1337 |
| TAA | | | 1340 |

(2) INFORMATION FOR SEQ ID NO:3:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 444 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(11) MOLECULE TYPE: protein

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 1 5 10 15
 Thr Val Lys Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
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 Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Asn
 35 40 45
 Ser Glu Asp Val His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
 50 55 60
 Ser Val Glu Ala Asp Lys Ala Ala Lys Arg Ala Val Val Val Gly Cys
 65 70 75 80
 Gly Gly Lys Phe Pro Val Glu Asp Ala Lys Glu Glu Val Gln Leu Phe
 85 90 95
 Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val Thr
 100 105 110
 Ala Ala Gly Gly Asn Ala Thr Tyr Val Leu Asp Gly Val Pro Arg Met
 115 120 125
 Arg Glu Arg Pro Ile Gly Asp Leu Val Val Gly Leu Lys Gln Leu Gly
 130 135 140
 Ala Asp Val Asp Cys Phe Leu Gly Thr Asp Cys Pro Pro Val Arg Val
 145 150 155 160
 Asn Gly Ile Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly Ser
 165 170 175
 Ile Ser Ser Gln Tyr Leu Ser Ala Leu Leu Met Ala Ala Pro Leu Ala
 180 185 190
 Leu Gly Asp Val Glu Ile Glu Ile Ile Asp Lys Leu Ile Ser Ile Pro
 195 200 205
 Tyr Val Glu Met Thr Leu Arg Leu Met Glu Arg Phe Gly Val Lys Ala
 210 215 220
 Glu His Ser Asp Ser Trp Asp Arg Phe Tyr Ile Lys Gly Gly Gln Lys
 225 230 235 240
 Tyr Lys Ser Pro Lys Asn Ala Tyr Val Glu Gly Asp Ala Ser Ser Ala
 245 250 255

Ser Tyr Phe Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Val Thr Val
 240 245 270
 Gly Gly Cys Gly Thr Thr Ser Leu Gln Gly Asp Val Lys Phe Ala Glu
 275 290 295
 Val Leu Glu Met Met Gly Ala Lys Val Thr Trp Thr Glu Thr Ser Val
 300 305
 Thr Val Thr Gly Pro Pro Arg Glu Pro Phe Gly Arg Lys His Leu Lys
 305 310 315 320
 Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr Leu
 325 330 335
 Ala Val Val Ala Leu Phe Ala Asp Gly Pro Thr Ala Ile Arg Asp Val
 340 345 350
 Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Val Ala Ile Arg Thr
 355 360 365
 Glu Leu Thr Lys Leu Gly Ala Ser Val Glu Glu Gly Pro Asp Tyr Cys
 370 375 380
 Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr Tyr
 385 390 395 400
 Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Ala Glu
 405 410 415
 Val Pro Val Thr Ile Arg Asp Pro Gly Cys Thr Arg Lys Thr Phe Pro
 420 425 430
 Asp Tyr Phe Asp Val Leu Ser Thr Phe Val Lys Asn
 435 440

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1340 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *See above*
- (B) STRAIN: Black Mexican Sweet

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pRPA-ML-720

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 6..1337

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

| | |
|---|-----|
| CCATG GGC GGC GAG GAG ATC GTG CTG CAG CCC ATC AAG GAG ATC | 47 |
| Ala Gly Ala Glu Glu Ile Val Leu Gln Pro Ile Lys Glu Ile | |
| 1 5 10 | |
| TCC GGC ACC GTC AAG GTG CCG GGG TCC AAG TCG CTT TCC AAC CGG ATC | 95 |
| Ser Gly Thr Val Lys Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile | |
| 15 20 25 30 | |
| CTC CTA CTC GGC GGC GTG TCC GAG GGG ACA ACA GTG GTT GAT AAC CTG | 143 |
| Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu | |
| 35 40 45 | |
| CTG AAC AGT GAG GAT GTC CAC TAC ATG CTC GGG GGC TTG AGG ACT CTT | 191 |
| Leu Asn Ser Glu Asp Val His Tyr Met Leu Gly Ala Leu Arg Thr Leu | |
| 50 55 60 | |
| GGT CTC TCT GTC GAA GCG GAC AAA GCT GCG AAA ABA GCT GTA GTT GTT | 239 |
| Gly Leu Ser Val Glu Ala Asp Lys Ala Ala Lys Arg Ala Val Val Val | |
| 65 70 75 | |

| | |
|---|------|
| GGC TTT GGT GGA AAT TTC GCA GTT GAG GAT GGT AAA GAG GAA GTG CAG Gly Cys Gly Gly Lys Phe Pro Val Glu Asp Ala Lys Glu Glu Val Gln 40 45 90 | 287 |
| CTC TTC TTT GAG AAT GCT GGA ATC GCA ATG GAT TCC TTT ACA GCA GCT Leu Phe Leu Gly Ala Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala 95 100 105 110 | 335 |
| GTT ACT GCT GCT GGT GGA AAT GCA ACT TAC GTG CTT GAT GGA GTA CCA Val Thr Ala Ala Gly Gly Asn Ala Thr Tyr Val Leu Asp Gly Val Pro 115 120 125 | 383 |
| AGA ATG AGG GAG AGA CCG ATT GGC GAC TTG GTT GTC GGA TTG AAG CAG Arg Met Arg Glu Arg Pro Ile Gly Asp Leu Val Val Gly Leu Lys Gln 130 135 140 | 431 |
| CTT GGT GCA GAT GTT GAT TGT TTC CTT GGC ACT GAC TGC CCA CCT GTT Leu Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asp Cys Pro Pro Val 145 150 155 | 479 |
| GCT GTC AAT GGA ATC GGA GGG CTA CCT GGT GGC AAG GTC AAG CTG TCT Arg Val Asn Gly Ile Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser 160 165 170 | 527 |
| GGC TCC ATC AGC AGT CAG TAC TTG AGT GGC TTG CTG ATG GCT GCT CCT Gly Ser Ile Ser Ser Gln Tyr Leu Ser Ala Leu Leu Met Ala Ala Pro 175 180 185 190 | 575 |
| TTG GCT CTT GGG GAT GTG GAG ATT GAA ATC ATT GAT AAA TTA ATC TCC Leu Ala Leu Gly Asp Val Glu Ile Glu Ile Ile Asp Lys Leu Ile Ser 195 200 205 | 623 |
| ATT CCG TAC GTC GAA ATG ACA TTG AGA TTG ATG GAG CGT TTT GGT GTG Ile Pro Tyr Val Glu Met Thr Leu Arg Leu Met Glu Arg Phe Gly Val 210 215 220 | 671 |
| AAA GCA GAG CAT TCT GAT AGC TGG GAC AGA TTC TAC ATT AAG GGA GGT Lys Ala Glu His Ser Asp Ser Trp Asp Arg Phe Tyr Ile Lys Gly Gly 225 230 235 | 719 |
| CAA AAA TAC AAG TCC CCT AAA AAT GCC TAT GTT GAA GGT GAT GCC TCA Gln Lys Tyr Lys Ser Pro Lys Asn Ala Tyr Val Glu Gly Asp Ala Ser 240 245 250 | 767 |
| AGC GCA AGC TAT TTC TTG GCT GGT GCT GCA ATT ACT GGA GGG ACT GTG Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Val 255 260 265 270 | 815 |
| ACT GTG GAA GGT TGT GGC ACC ACC AGT TTG CAG GGT GAT GTG AAG TTT Thr Val Glu Gly Cys Gly Thr Thr Ser Leu Gln Gly Asp Val Lys Phe 275 280 285 | 863 |
| GCT GAG GTA CTG GAG ATG ATG GGA GCG AAG GTT ACA TGG ACC GAG ACT Ala Glu Val Glu Met Met Gly Ala Lys Val Thr Trp Thr Glu Thr 290 295 300 | 911 |
| AGC GTA ACT GTT ACT GGC CCA CCG CAG GAG CCA TTT GGG AGG AAA CAC Ser Val Thr Val Thr Gly Pro Pro Arg Glu Pro Phe Gly Arg Lys His 305 310 315 | 959 |
| CTC AAG GCG ATT GAT GTC AAC ATG AAC AAG ATG CCT GAT GTC GCC ATG Leu Lys Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met 320 325 330 | 1007 |
| ACT CTT GCT GTG GTT GCG CTC TTT GCG GAT GGC CCG ACA GCC ATC AGA Thr Leu Ala Val Val Ala Leu Phe Ala Asp Gly Pro Thr Ala Ile Arg 335 340 345 350 | 1055 |
| GAC GTG GCT TCC TGG AGA GTA AAG GAG ACC GAG AAG ATG GTT GCG ATC Asp Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Val Ala Ile 355 360 365 | 1103 |
| GGG ACG GAG CTA ACC AAG CTG GGA GCA TCT GTT GAG GAA GGG CCG GAC Arg Thr Glu Leu Thr Lys Leu Gly Ala Ser Val Glu Glu Gly Pro Asp 370 375 380 | 1151 |
| TAC TGC ATC ATC ACG CCG CCG GAG AAG CTG AAC GTG ACG GCG ATC GAC Tyr Cys Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp 385 390 395 | 1199 |

ACT GAC GAC CAC CAG AAG ATG GCG ATG GCG TTC TCG CTT GCG GCG TGT 1247
 Thr Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys
 100 105 110
 GCG GAG GTC CCG GTC ACC ATC GCG GAC GGT GAG TGC ACC GCG AAG ACC 1295
 Ala Glu Val Pro Val Thr Ile Arg Asp Pro Gly Cys Thr Arg Lys Thr
 115 120 125 130
 TTC CCG GAC TAC TTC GAT GTG CTG AGC ACT TTC GTC AAG AAT 1337
 Phe Pro Asp Tyr Phe Asp Val Leu Ser Thr Phe Val Lys Asn
 135 140
 TAA 1340

(2) INFORMATION FOR SEQ ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 444 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(11) MOLECULE TYPE: protein

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Gly Ala Glu Glu Ile Val Leu Gln Pro Ile Lys Glu Ile Ser Gly 15
 1 5 10
 Thr Val Lys Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu 30
 20 25 30
 Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Asn 45
 35 40 45
 Ser Glu Asp Val His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu 60
 50 55 60
 Ser Val Glu Ala Asp Lys Ala Ala Lys Arg Ala Val Val Val Gly Cys 80
 65 70 75 80
 Gly Gly Lys Phe Pro Val Glu Asp Ala Lys Glu Glu Val Gln Leu Phe 95
 85 90 95
 Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val Thr 110
 100 105 110
 Ala Ala Gly Gly Asn Ala Thr Tyr Val Leu Asp Gly Val Pro Arg Met 125
 115 120 125
 Arg Glu Arg Pro Ile Gly Asp Leu Val Val Gly Leu Lys Gln Leu Gly 140
 130 135 140
 Ala Asp Val Asp Cys Phe Leu Gly Thr Asp Cys Pro Pro Val Arg Val 160
 145 150 155 160
 Asn Gly Ile Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly Ser 175
 165 170 175
 Ile Ser Ser Gln Tyr Leu Ser Ala Leu Leu Met Ala Ala Pro Leu Ala 190
 180 185 190
 Leu Gly Asp Val Glu Ile Glu Ile Ile Asp Lys Leu Ile Ser Ile Pro 205
 195 200 205
 Tyr Val Glu Met Thr Leu Arg Leu Met Glu Arg Phe Gly Val Lys Ala 220
 210 215 220
 Glu His Ser Asp Ser Trp Asp Arg Phe Tyr Ile Lys Gly Gly Gln Lys 240
 225 230 235 240
 Tyr Lys Ser Pro Lys Asn Ala Tyr Val Glu Gly Asp Ala Ser Ser Ala 255
 245 250 255
 Ser Tyr Phe Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Val Thr Val 270
 260 265 270
 Glu Gly Cys Gly Thr Thr Ser Leu Gln Gly Asp Val Lys Phe Ala Glu 285
 275 280 285

Val Leu Glu Met Met Gly Ala Lys Val Thr Trp Thr Glu Thr Ser Val
 290 295 300
 Thr Val Thr Gly Pro Pro Arg Glu Pro Phe Gly Arg Lys His Leu Lys
 305 310 315 320
 Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr Leu
 325 330 335
 Ala Val Val Ala Leu Phe Ala Asp Gly Pro Thr Ala Ile Arg Asp Val
 340 345 350
 Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Val Ala Ile Arg Thr
 355 360 365
 Glu Leu Thr Lys Leu Gly Ala Ser Val Glu Glu Gly Pro Asp Tyr Cys
 370 375 380
 Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr Tyr
 385 390 395 400
 Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Ala Glu
 405 410 415
 Val Pro Val Thr Ile Arg Asp Pro Gly Cys Thr Arg Lys Thr Phe Pro
 420 425 430
 Asp Tyr Phe Asp Val Leu Ser Thr Phe Val Lys Asn
 435 440

CLAIMS

1. DNA gene coding for a mutated
5-enolpyruvylshikimate-3-phosphate synthase (EPSPS),
characterized in that it comprises at least one
5 threonine 102 → isoleucine substitution.
2. DNA gene according to claim 1,
characterized in that it comprises, in addition, at
least a second mutation in the EPSPS, different from
the first mutation.
- 10 3. DNA gene according to claim 2,
characterized in that it comprises, in addition, a
mutation consisting of a substitution of proline 106 by
serine.
- 15 4. DNA gene according to claim 2,
characterized in that it comprises, in addition, a
mutation consisting of a substitution of glycine 101 by
alanine.
5. DNA gene according to one of claims 1 to
4, characterized in that it is of bacterial origin.
- 20 6. DNA gene according to claim 5,
characterized in that it originates from a bacterium of
the genus *Salmonella typhimurium*.
7. DNA gene according to one of claims 1 to
4, characterized in that it is of plant origin.
- 25 8. DNA gene according to claim 7,
characterized in that it is of maize origin.
9. Mutated EPSPS protein, characterized in
that it comprises at least one substitution of

threonine 102 by isoleucine.

10. Chimeric gene comprising a coding
sequence as well as regulatory elements at positions 5'
and 3' which are heterologous and capable of
5 functioning in plants, characterized in that it
comprises as coding sequence at least one sequence
according to one of claims 1 to 8.

11. Chimeric gene according to claim 9,
characterized in that it comprises a plant virus
10 promoter.

12. Chimeric gene according to claim 10,
characterized in that it comprises a plant promoter
(e.g. α -tubulin, histone, introns, actin, etc.).

13. Vector for the transformation of plants,
15 characterized in that it comprises at least one gene
according to one of claims 10 to 12.

14. Plant cell, characterized in that it
comprises at least one gene according to one of claims
10 to 12.

20 15. Plant, characterized in that it is
obtained by regeneration from a cell according to claim
14.

16. Method for the production of plants with
improved tolerance to a herbicide having EPSP synthase
25 as its target, characterized in that plant cells or
protoplasts are transformed with a gene according to
one of claims 1 to 8, and in that the transformed cells
are subjected to a regeneration.

17. Method of treatment of plants with a herbicide having EPSPS as its target, characterized in that the herbicide is applied to plants according to claim 15.

5 18. Method according to claim 17, characterized in that glyphosate or a glyphosate precursor is applied.

COMBINED DECLARATION AND POWER OF ATTORNEY

Attorney Docket No. 5500*13
RP/PCT-1

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of subject matter which is claimed and for which a patent is sought on the invention entitled
MUTATED 5-ENOL PYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE, GENE CODING FOR SAID PROTEIN AND TRANSFORMED PLANTS CONTANING SAID GENE

the specification of which

(check one) ☐ is attached hereto.

☒ was filed on October 14, 1997 as ☒ Application Serial No. 08/945,144

☐ Express Mail No. _____, as Serial No. not yet known,
and including all the amendments through the date hereof.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Priority Claimed

PCT/FR96/01125
(Number)

PCT
(Country)

18/7/96
(Day/Month/Year Filed) ☒ ☐
Yes No

95/08979
(Number)

France
(Country)

19/7/95
(Day/Month/Year Filed) ☒ ☐
Yes No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112. I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

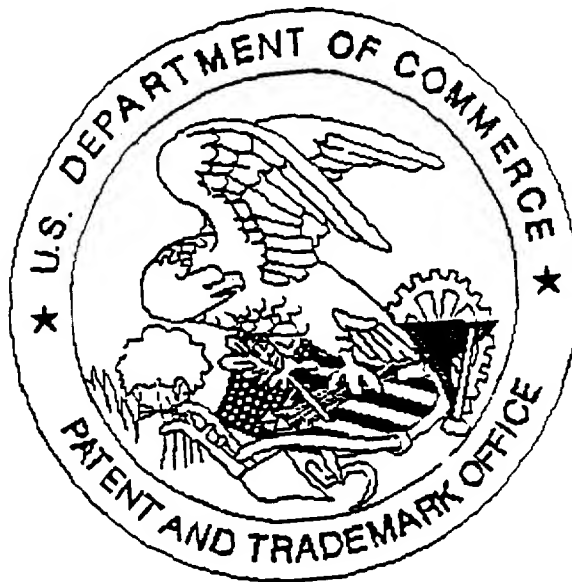
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

13- Rudolf E. Hutz, Reg. No. 22,397; Harold Pezzner, Reg. No. 22,112; John D. Fairchild, Reg. No. 19,756; Richard M. Beck, Reg. No. 22,580; Paul E. Crawford, Reg. No. 24,397; Thomas M. Meshbesh, Reg. No. 25,083; Patricia Smink Rogowski, Reg. No. 33,791; Robert G. McMorrow, Jr., Reg. No. 30,962; Ashley I. Pezzner, Reg. No. 35,646; William E. McShane, Reg. No. 32,707; Mary W. Bourke, Reg. No. 30,982; Gerard M. O'Rourke, Reg. No. 39,794; Allan N. Kutzenko, Reg. No. 38,945, all of P.O. Box 2207, Wilmington, Delaware 19899-2007, my attorneys with full power of substitution and revocation.

| | | |
|---|--|--|
| Send Correspondence To: Connolly and Hutz P.O. Box 2207 Wilmington, Delaware 19899-2207 | | Direct Telephone Calls To: (302) 658-9141 |
| 1-00 | | |
| FULL NAME OF FIRST INVENTOR MICHEL LEBRUN | INVENTOR'S SIGNATURE <i>Michel Lebrun</i> | DATE December, 11th 1997 |
| RESIDENCE 224 Rue de Saint-Cyr, 69009 LYON, France | FLX | CITIZENSHIP France |
| POST OFFICE ADDRESS 224 Rue de Saint-Cyr, 69009 LYON, France | | |
| 2-00 | | |
| FULL NAME OF SECOND JOINT INVENTOR IF ANY ALAIN SAILLAND | INVENTOR'S SIGNATURE <i>Alain SAILLAND</i> | DATE December, 16th 1997 |
| RESIDENCE 38 Rue Albert Chalinel, 69009 LYON, France | | CITIZENSHIP France |
| POST OFFICE ADDRESS 38 Rue Albert Chalinel, 69009 LYON, France | | |
| 3-00 | | |
| FULL NAME OF THIRD JOINT INVENTOR IF ANY GEORGES FREYSSINET | INVENTOR'S SIGNATURE <i>Georges Freysinet</i> | DATE December 15th, 1997 |
| RESIDENCE 21 Rue de Nervieux, 69450 ST CYR AU MONT D'OR, France | FLX | CITIZENSHIP France |
| POST OFFICE ADDRESS 21 Rue de Nervieux, 69450 ST CYR AU MONT D'OR, France | | |
| 4-00 | | |
| FULL NAME OF FOURTH JOINT INVENTOR IF ANY ERIC DEGRYSE | INVENTOR'S SIGNATURE <i>Eric Degryse</i> | DATE December, 27th 1997 |
| RESIDENCE 4 Rue des Alisiers, 67100 Strasbourg, France | FAX | CITIZENSHIP France |
| POST OFFICE ADDRESS 4 Rue des Alisiers, 67100 Strasbourg, France | | |
| 5-00 | | |
| FULL NAME OF FIFTH JOINT INVENTOR IF ANY | INVENTOR'S SIGNATURE | DATE |
| RESIDENCE | | CITIZENSHIP |
| POST OFFICE ADDRESS | | |
| 6-00 | | |
| FULL NAME OF SIXTH JOINT INVENTOR IF ANY | INVENTOR'S SIGNATURE | DATE |
| RESIDENCE | | CITIZENSHIP |
| POST OFFICE ADDRESS | | |

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



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